

INFLUENCE OF EXOGENOUS FACTORS ON THE FORMATION OF DAILY MICROGROWTH INCREMENTS IN OTOLITHS OF *TILAPIA MARIAE* (BOULENGER, 1899) JUVENILES

by

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SUMMARY. — The influence of photoperiod, of feeding frequency and of a water temperature cycle on the formation of daily microgrowth increments was studied in lapilli of juvenile *Tilapia mariae*. These exogenous factors did not affect the production of daily increments, suggesting the existence of a circadian endogenous rhythm. The characteristics of the increments were affected by the experimental conditions, suggesting that the endogenous rhythm promotes a circadian cycle of activity or rest, which results in the formation of daily increments.

RÉSUMÉ. — L'influence de la photopériode, de la fréquence de prise de nourriture et du cycle de la température de l'eau sur la formation des microstructures de croissance des otolithes (lapilli) a été étudiée chez les juvéniles de *Tilapia mariae*. Ces facteurs externes n'affectent pas la formation des stries d'accroissement journalières, suggérant ainsi l'existence d'un rythme circadien interne. Les caractéristiques des stries d'accroissement ont été modifiées par les conditions expérimentales, ce qui laisse penser que le rythme interne entraîne un cycle circadien d'activité et de repos, dont le résultat est la formation de stries d'accroissement journalières.

Key-words : Pisces, *Tilapia mariae*, Cichlidae, circadian rhythms, growth, otolith reading, experimental data.

INTRODUCTION

Since the pioneer work of Pannella (1971, 1974), daily microgrowth increments of fish otoliths have been widely used and investigated, mainly in larvae and juveniles. Several authors investigated the influence of exogenous factors in the deposition of such increments, photoperiod, temperature and feeding frequency, being the factors usually tested. The results obtained in this area of research have often been quite different, and in some cases have led to contradictory conclusions.

Taubert & Coble (1977) concluded that in young *Tilapia mossambica* (= *Sarotherodon mossambicus*), there is an internal circadian rhythm (biological clock) which is responsible for the deposition of daily increments, needing a 24 hour photoperiod to function. Temperature and feeding frequency did not affect the periodicity of the increments. Similar conclusions were reached by Tanaka *et al.*

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(1981) for juveniles of *Tilapia nilotica*. Neilson & Geen (1982) found evidence of the existence of a biological clock controlling daily increment deposition, affected by feeding frequency and not by photoperiod or temperature, in juveniles of *Oncorhynchus tshawytscha*. The existence of an endogenous circadian rhythmicity not affected by environmental variables was suggested by Campana & Neilson (1982) for juveniles of *Platichthys stellatus*. Brothers (1981) mentioned temperature as the factor responsible for the periodicity of the increments and attributed subordinate roles to light and food, in temperate stream populations. Pannella (1980) referred the existence of a close relationship between feeding frequency and the number of increments deposited per day in *Tilapia* sp. Radtke & Dean (1982) concluded that, in embryos and larvae of *Fundulus heteroclitus*, light initiates increment formation, which is controlled by a 24-hour photoperiod. Marshall & Parker (1982) showed that increment formation ceases at low temperatures, that feeding frequency is not a significant factor and that photoperiod is probably important in juveniles of *Oncorhynchus nerka*. Campana (1983) showed that feeding frequency does not affect the daily periodicity of the increments, in juveniles of *Salmo gairdneri* and *Platichthys stellatus*. Geffen (1982) concluded that, in larval *Clupea harengus* and *Scophthalmus maximus*, increment periodicity depends on the individual growth rate (being under indirect influence of environmental factors), and is daily only at certain growth rates. In embryos of *Salmo salar*, the same author (Geffen, 1983) showed that otolith increments are not deposited on a daily basis, and that their deposition is affected by photoperiod and temperature, possibly due to the influence of these factors on physiological activity.

It is obvious that the reasons underlying the deposition of daily microgrowth increments in fish otoliths are far from being elucidated. An understanding of such reasons is important to validate the use of otolith microstructure in several areas of research, mainly those concerned with age and growth.

The objective of this paper is to contribute to the knowledge of the influence of environmental factors in the production of otolith daily microgrowth increments. Photoperiod, feeding frequency and temperature were the factors tested, in juveniles of the spotted tilapia, *Tilapia mariae*.

MATERIAL AND METHODS

A couple of adult *Tilapia mariae* was kept in a 200 l aquarium, together with some adults of *Sarotherodon mossambicus*, under a photoperiod of 12 h of light and 12 h of darkness (light phase from 08.00 h to 20.00 h), and at a temperature of $26.5 \pm 0.5^\circ\text{C}$. This couple spawned regularly once per month, with the female laying the eggs (around 250 to 350 per spawning) on an artificial substrate. For experimental purposes only one spawning (around 300 eggs) was used. Shortly after fertilization, the eggs were transferred attached to the substrate to a 50 l aquarium under the same photoperiod, at a water temperature of $26.0 \pm 1.5^\circ\text{C}$ (not varying in a diel fashion). Hatching occurred 2 days after fertilization between

16.00 h and 17.00 h. Swimming up was reached between day 5 and day 6 after hatching. First feeding was provided in excess at 16.00 h on day 7, when mouth and digestive tract were fully developed and there was still vestigial yolk in the abdomen. Excess food was siphoned one hour later. By day 8 the yolk-sac was completely absorbed, being the young *T. mariae* considered juveniles thereafter. On that day a second feeding was given in the same way as the first one, and at 17.00 h the juveniles were placed in five 50 l aquaria submitted to different experimental conditions. The experimental conditions were conceived to test the influence of photoperiod, feeding frequency and of a circadian cycle of temperature on the deposition of daily microgrowth increments. The five experiments undertaken were :

- (1) Constant light (LK); temperature of $25.5 \pm 1.5^{\circ}\text{C}$, not varying in a diel basis (TK); feeding once every 6 h, at 04.00 h, 10.00 h, 16.00 h and 22.00 h (F 1/6). If daily increments would form under these conditions, in the absence of any circadian stimulus of exogenous factors, we would conclude to the existence of a biological clock determining their deposition.
- (2) LK; TK; feeding once per day, at 16.00 h (F 1/24). This experiment would test whether a daily feeding regime could promote daily increment deposition.
- (3) LK; temperature varying in a diel fashion, from a maximum of $29-30^{\circ}\text{C}$ at 20.00 h, to a minimum of $23-25^{\circ}\text{C}$ at 08.00 h (TV); F 1/6. The factor tested would be a circadian cycle of temperature.
- (4) Photoperiod of 12 hours of light and 12 h of darkness, with the light phase from 08.00 to 20.00 h (L12:D12); TK; F 1/6. This experiment would test the influence of a 24 h photoperiod. However, the fact that two feedings per day (those at 04.00 h and 22.00 h) would coincide with the dark phase could interfere with the interpretation of the experimental results, due to the possibility that the juveniles would not eat without light.
- (5) L12:D12; TK; feeding once every 48 h, at 16.00 h (F 1/48). This experiment would also test a circadian photoperiod, as the other factors would not vary in a diel basis. If feeding frequency would determine the periodicity of the increments, once every two days would be expected.

All the feeding consisted of a commercial dry food for tropical fish, reduced to powder. The meals of experiments 1, 3 and 4 (F 1/6) were provided in excess by automatic feeders, roughly the same quantities in each meal. The meals of experiments 2 and 5 (F 1/24 and F 1/48) were given manually, in excess and in approximately equal quantities. The feeding frequencies established for each experiment could be falsified if excess food would remain available to the individuals between the meals, specially in experiments 2 and 5. To prevent this a method similar to the one used by Taubert & Coble (1977) was used : in each aquarium the juveniles were held in an aerated plastic container with a screen bottom, whose mesh size was smaller than the individuals and larger than the food particles, in such a way that food was only available during the few minutes it took to sink. Three to four times a week excess food was siphoned from the bottom of the aquaria. The lighting was provided by fluorescent « gro-lux » strip lamps (20 W), with

each lamp placed over the glass covers of two contiguous aquaria, around 6 cm above the water surface. Photoperiod was controlled automatically by programmable clocks, without a dusk or dawn transition in light intensity. The temperature cycle of experiment 3 was obtained by the use of a programmable clock controlling an electrical resistance, which functioned from 08.00 h to 20.00 h. In the other cases, temperature was maintained by the use of thermostats and electrical resistances. Water temperature ($\pm 0.1^{\circ}\text{C}$) was measured several times per day with an alcohol thermometer.

Each experiment began with a total of 40 juveniles. Some juveniles (around 20) were kept under the same conditions under which the larvae were reared, with exogenous daily feeding (L12:D12, TK, F 1/24). They were also held in a plastic container, and they were fed manually in excess. Part of the individuals of experiment 2 (LK, TK, F 1/24) were submitted to a parallel experiment: on day 21, 10 individuals were separated to another plastic container in the same aquaria, and their feeding regime was changed to one meal per 48 h, with food provided manually in excess at 16.00 h from day 22. This experiment will be referred to as experiment 2-A (LK, TK, F 1/24 + F 1/48).

Several embryos or larvae were sacrificed daily from fertilization to yolk-sac absorption, for observation under a binocular microscope and for otolith extraction. The juveniles which remained under L12:D12 and TK, with F 1/24, were sacrificed during the first 10 days after the beginning of the experiments. The juveniles of the experiments were sampled between 14.00 h and 17.00 h with the following frequencies:

- experiments 1, 3 and 4: 5 individuals on days 18, 21 and 25; 10 on day 28; 15 on day 32 (total: 40).
- experiment 2: 5 on days 18 and 25; 10 on days 28 and 33 (total: 30).
- experiment 2-A: all (10) on day 28.
- experiment 5: 5 on days 18, 21 and 25; 10 on day 28; 13 on day 33 (total: 38; 2 died naturally during the experimental period and were discarded).

Both standard length (± 0.15 mm) and fresh weight (± 0.1 mg) were measured for each juvenile immediately after death. The individuals were dissected using fine forceps and needles, under a magnification of 16 or 40 X with the aid of a binocular microscope. Our previous observations of *T. mariae* larvae and juveniles showed that utricular otoliths (lapilli) were the best suited for this study. The sagittae soon become too thick to allow the observation of their microstructure without being ground, and the asterisci microgrowth increments are very narrow, unclear and difficult to enumerate. The lapilli remain thin enough, at least during the time of the experiments, so that their microstructural increments can be easily seen under transmitted light without special preparation. After extraction the lapilli were made free of involving tissues and were placed in a drop of water on a glass slide. After drying they were whole mounted in DePeX and viewed with the aid of a light microscope (magnifications of 600 or 1250 X). The mounting medium used (DePeX) did not seem to affect the clearness of the increments, since there was no noti-

ceable difference in otoliths viewed immediately after mounting and up to until one month later. The lapillus of *T. mariae* has approximately the form of an oval disc. Maximum diameter ($\pm 1.15 \mu\text{m}$) of both lapilli of each fish was measured with a calibrated eyepiece, and the mean was used for data analysis. We found no significant differences between the diameters of the two sides ($t = 1.824$, d.f. = 175, $P > 0.05$). When one of the otoliths was unsuitable for measurement due to any reason (broken, abnormal, etc.) the value of the other was used.

As most authors define it, one microgrowth increment is a bipartite structure composed of two adjacent zones, which under transmitted light appear as a narrow and dark one, and a wider and translucent one. Similarly to Campana & Neilson (1982), we distinguished two kinds of increments (major and minor), based on their relative intensity, width, regularity of spacing, and continuity. Major increments were more intense, wider, more or less regularly spaced, continuous along the otolith and often composed of several minor increments. The latter were fainter, narrower and often irregular, which made them difficult to enumerate. Total counts were made only of major increments, although we estimated the number of minor increments per major increment in each experiment. The increments were enumerated on the posterior part of the lapillus, where they were wider and more discernible. Counts were initiated from the major increment corresponding to the day of the beginning of the experiments (see Results). Several counts were made for each lapillus. Both left and right lapillus of each fish were examined, and for data analysis the mean of both sides was used. There were no significant differences between the counts of the two sides ($t = 1.238$, d.f. = 123, $P > 0.05$). When one of the otoliths was unsuitable for examination due to some reason (abnormal otolith, unclear microstructure, deficiency in the preparation process) only the other was used. Of the 198 experimental juveniles, 30 had both otoliths unsuitable for increment enumeration and were discarded.

RESULTS

Two pairs of otoliths were present in the otic capsule of *T. mariae* embryos from about one day before hatching. At this stage it was not possible to know which otoliths were these first pairs. However the daily observation of the larvae and of the development of the inner ear revealed that the anterior ones were the lapilli and the posterior were the sagittae. The asterisci were formed later, one or two days before the complete yolk-sac absorption, near the sagittae, and are the smaller pair of otoliths. Lapillus formation starts from one to several spherules (primordia) which grow into contact with one another. The lapillus grows by deposition of material around the primordia, which constitute the core of the otolith. No increments were formed during the embryonic period. It has been suggested that larvae which have long incubation periods form increments before hatching, while larvae which have short incubation periods do not (Radtke & Waiwood, 1980; Radtke & Dean, 1982). *T. mariae* larvae, which have a very short incubation period (about 2 days under the rearing conditions described) meet this suggestion.

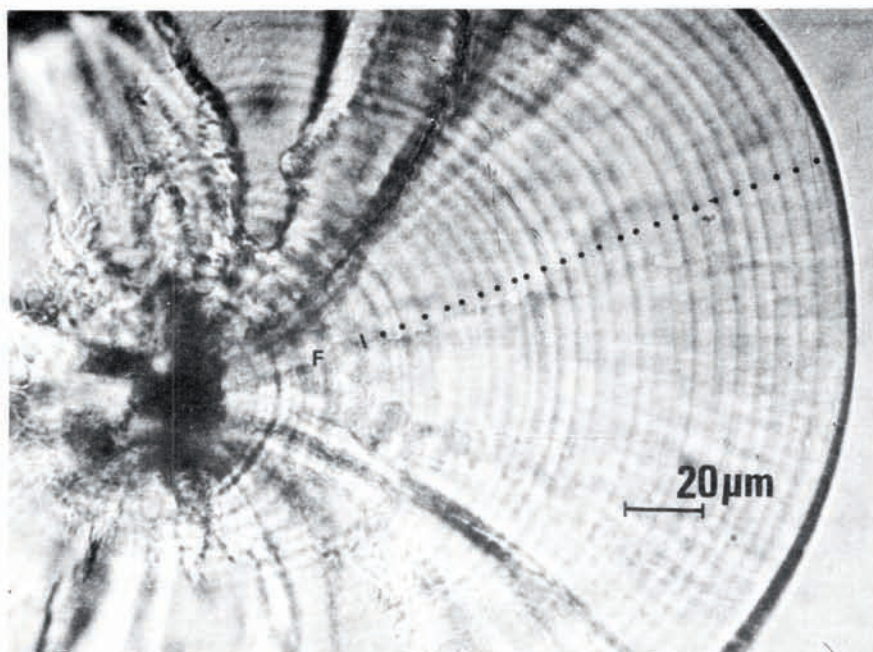


Fig. 1. — Lapillus of a 33 days-old juvenile from experiment 2 (LK, TK, F1/24), showing 25 major increments since the beginning of the experiments (—). (F) First-feeding check.

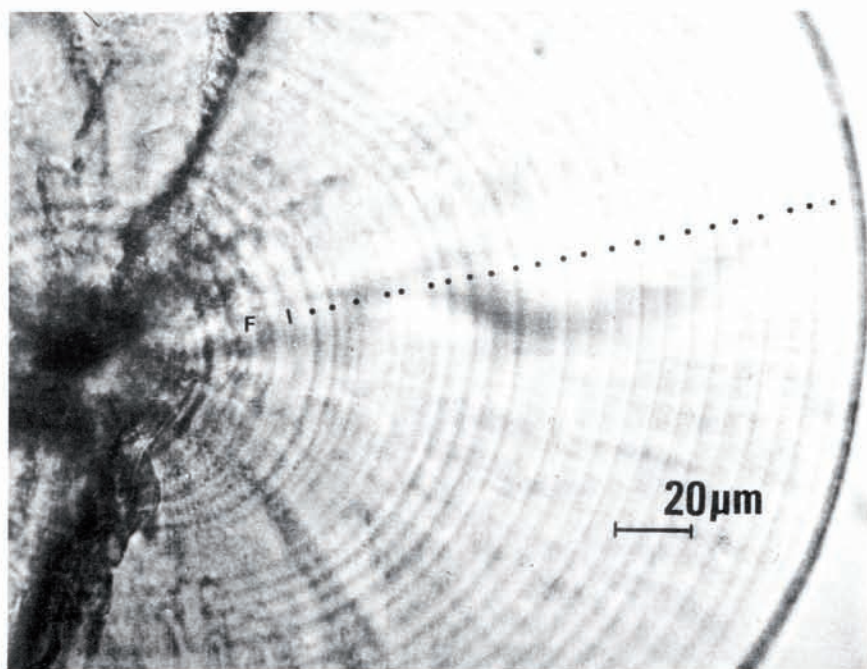


Fig. 2. — Lapillus of a 32 days-old juvenile from experiment 4 (L12 : 012, TK, F1/6), showing 24 major increments since the beginning of the experiments (—). (F) First-feeding check.

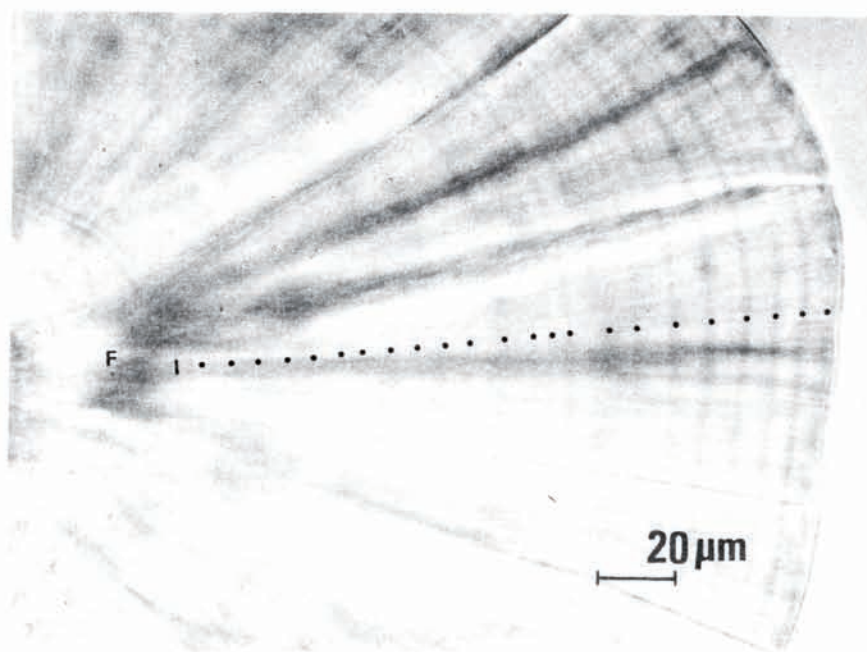


Fig. 3. — Lapillus of a 32 days-old juvenile from experiment 1 (LK, TK, F1/6), showing 24 major increments since the beginning of the experiments (—), being the last ones more discernible (F) First-feeding check

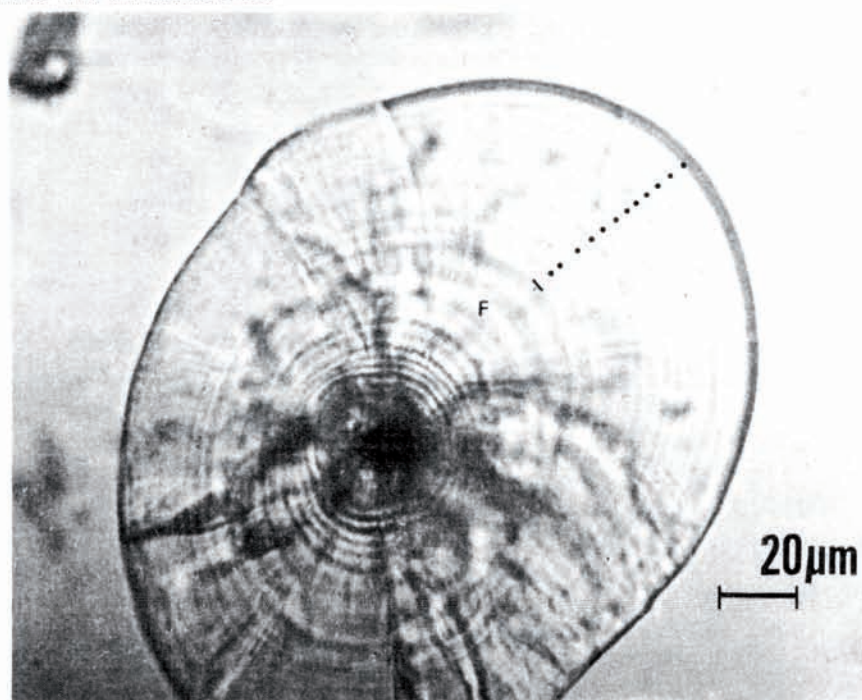


Fig. 4. — Lapillus of a 21 days-old juvenile from experiment 5 (L12 : D12, TK, F1/48), showing 13 major increments since the beginning of the experiments (—). It can be noticed that some increments, corresponding to days when food was provided, are slightly more intense. (F) First-feeding check.

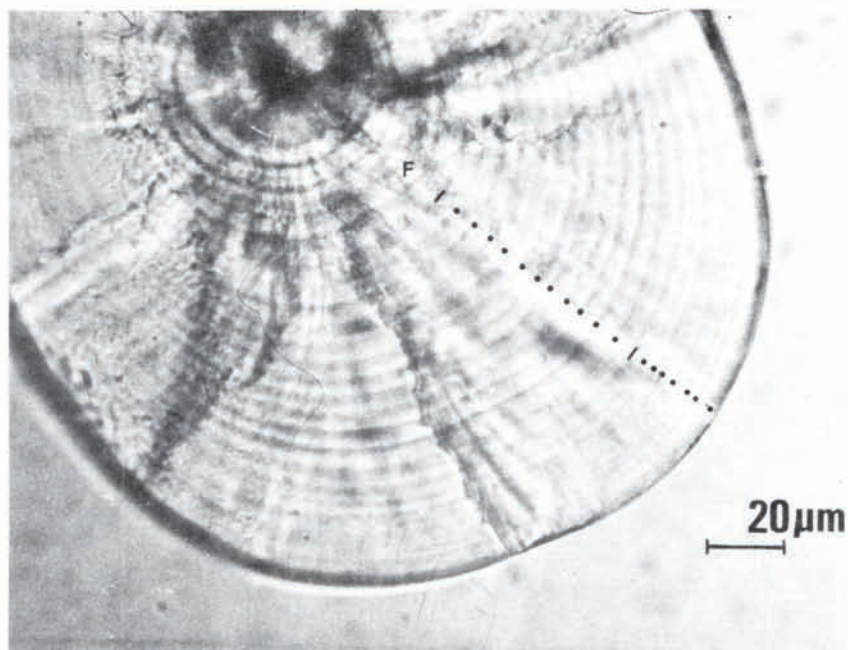


Fig. 5. — Lapillus of a 28 days-old juvenile from experiment 2-A (LK, TK, F1/24 + F1/48), showing 20 major increments since the beginning of the experiments (first bar). The increments became narrower and fainter after the changing of the feeding regime (second bar). (F) First-feeding check.

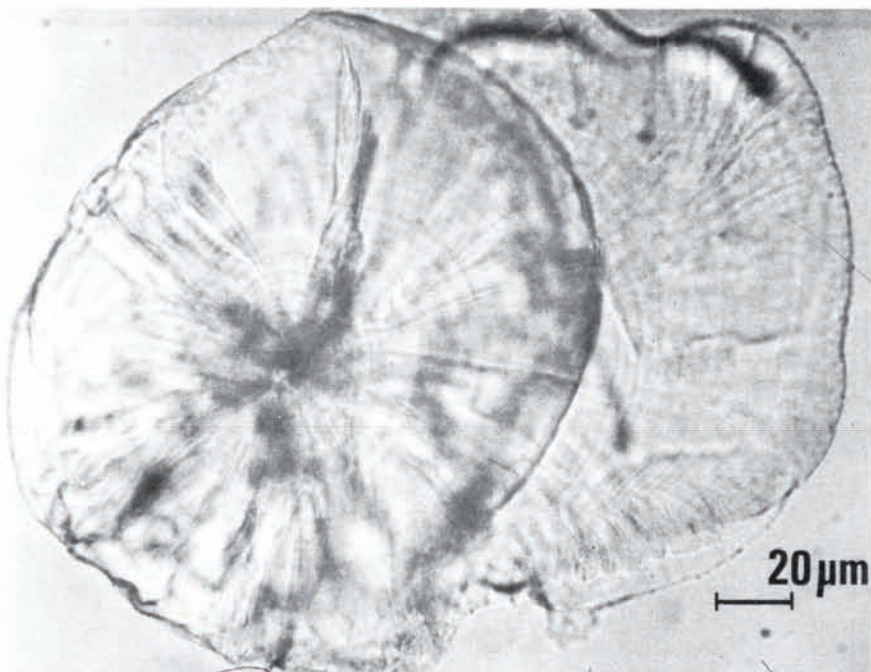


Fig. 6. — Lapillus with an abnormal structure, seeming composed of two kinds of materials. Major increments, although present in both parts, are not clear enough to be enumerated.

Microstructural increment deposition started at day 2. From this day to day 4, two narrow increments were formed per day, without any clear distinction between major and minor increments. The two kinds of increments could be distinguished since day 5. From this day to day 8 (beginning of the experiments) one major increment, divided into two to three minor ones, was formed per day. Brothers *et al.* (1976) showed that the exact timing of the initiation of daily increment formation varies from species to species. In *T. mariae* it seems that major increments start forming on a daily basis since swimming-up, which occurred at day 5. The juveniles kept under the same rearing conditions of the larvae, and with daily feeding, went on forming one major increment per day in their lapilli.

In all the individuals studied, the major increment deposited on day 7 was wider than the preceding ones and was divided into three minor increments. The translucent zone of one of these had optical properties which made it distinct from the other increments; it was lighter, and was clearer at a different focal depth than the others. We associated this check with first feeding, which was provided at day 7. Several authors have associated checks in larval and juvenile otoliths to different events of the life cycle. Marshall & Parker (1982) also associated a check with first feeding, or to the transition period from passive yolk-absorption to active foraging in *Oncorhynchus nerka*. The increment pattern deposited during the larval period was very constant in all individuals, particularly the first-feeding check, which was always detectable. Counts were made from the increment correspondent to the day of the beginning of the experiments (day 8). This increment was easily detectable due to the first-feeding check and to the constancy of the increment pattern of the larval period. The major increments formed after the complete yolk-sac absorption were usually more regular in spacing and intensity than the previous ones.

The slopes of the regressions of increment counts on days since the beginning of the experiments (Table II) were not significantly different from 1 at the 95 % confidence level for experiments 2, 3, 4 and 5. For experiment 1 the difference was significant at the 95 % but not at the 99 % level (t-test); in most of the individuals of this experiment there was only one increment less than the number of days (Table I). For experiment 2-A the mean number of increments did not differ significantly from the number of days elapsed (t-test, $P > 0.05$). This indicates that major increments were formed on a daily basis in all experiments. Minor increments, which when present were several per major increment, were consequently deposited on a subdaily basis.

However the experimental conditions had clearly an effect on the characteristics of otolith microstructure. In experiments 2 (LK, TK, F 1/24) and 4 (L12:D12, TK, F 1/6) the major increments were generally intense, easily distinguished from minor increments (2 or 3 per major increment) and easily enumerated (Figures 1 and 2), reasons which probably explain the very low standard deviations verified in increment number (Table I). In experiments 1 (LK, TK, F 1/6) and 3 (LK, TV, F 1/6) major increments were wider, fainter, difficult to enumerate, and with 3 to 4 minor increments each (Figure 3). The general faintness of the increments pattern can probably account for some of the « lost » increments in the counts of these experiments (Table I). In experiment 5 (L12:D12, TK, F 1/48) major increments were very narrow, intermediate in intensity comparatively with experiments 2-4

Table I. — Mean number of major microgrowth increments in the different experiments. D - number of days elapsed since the beginning of the experiments; I - mean major increment number; SD - standard deviation; n - number of individuals examined; n' - number of individuals discarded.

Experiment	D	I	SD	n	n'	
1	10	9.50	0.71	2	3	
LK, TK,	13	12.75	0.35	2	3	
F 1/6	17	17.00	0.00	5	0	
	20	19.30	1.00	9	1	
	24	23.00	0.00	12	3	Total :
				30	10	40
2	10	10.00	0.00	5	0	
LK, TK,	17	17.00	0.00	5	0	
F 1/24	20	20.00	0.00	9	1	
	25	24.70	0.48	10	0	Total :
				29	1	30
2-A	20	19.44	1.49	9	1	Total :
LK, TK						10
F 1/24 + 1/48						
3	10	10.00	0.00	4	1	
LK, TV,	13	12.70	0.45	5	0	
F 1/6	17	15.90	1.14	5	0	
	20	19.25	0.35	10	0	
	24	23.36	0.48	7	8	Total :
				31	9	40
4	10	10.00	0.00	4	1	
L12: D12,	13	12.88	0.25	4	1	
TK, F 1/6	17	17.00	0.00	5	0	
	20	20.00	0.00	10	0	
	24	24.00	0.00	15	0	Total :
				38	2	40
5	10	9.88	0.25	4	1	
L12: D12,	13	11.38	1.60	4	1	
TK, F 1/48	17	15.75	0.64	4	1	
	20	19.72	1.00	9	1	
	25	24.00	1.20	9	4	Total :
				30	8	38

Table II. — Regression coefficients (b), y-intercepts (a) and correlation coefficients (r) for regressions of major increment counts on days elapsed since the beginning of the experiments. (t) t- test value for the comparison of (b) with 1; (d.f. degrees of freedom; (n) number of individuals.

Experiment	n	b	a	r	t	d. f.
1	30	0.934	1.188	0.989	2.510	28
2	29	0.986	0.316	0.999	1.860	27
3	31	0.957	0.499	0.992	1.841	29
4	38	1.004	- 0.114	0.999	1.354	36
5	30	0.989	- 0.508	0.978	0.274	28

and 1-3, and minor increments were practically absent or indistinct (Figure 4). An interesting feature observed in many of the otoliths of experiment 5 was the slight increase in intensity of the increments corresponding to days when meals were provided (Figure 4). This pattern was lacking in some otoliths, and when present it was usually not noticeable in whole otolith. A similar subtle pattern was detected by Campana (1983) in juvenile *Salmo gairdneri* fed once every three days. In experiment 2-A (LK, TK, F 1/24 + F 1/48), major increments became noticeably narrower and fainter in relation to the preceding ones, since the change of the feeding regime, and minor increments had a drastical decrease in number (Figure 5). In several otoliths of this experiment we detected the same subtle pattern of relative intensities, corresponding to the presence or absence of a meal on the respective day.

Some lapilli had a microstructure such that the growth increments, although present, were too faint and unclear to be counted. In many of these cases there were patterns of lines irradiating from the core of the otolith that made microgrowth increments indistinct. These otoliths constituted around 35 % of the otoliths in experiments 1 and 5, 25 % in experiment 3, and only about 5 % in experiments 2 and 4. Unclear microstructure seemed to be related somehow to the general faintness of the increments in experiments 1 and 3, or to their narrowness in experiment 5. Other otoliths showed an abnormal structure, as if their constituent material became different after a certain moment. Fish otoliths are composed of a protein (otolin) and of calcium carbonate, usually in the form of aragonite (Degens *et al.*, 1969). Taking into account the description that Campana (1983) made of vateritic otoliths (« altered structure and glasslike appearance »), vaterite, which is an alternate form of calcium carbonate, was possibly the different mineral form of these otoliths, which would then be partially aragonitic and partially vateritic. Although microgrowth increments were present in both parts of these otoliths (Figure 6), they were too unclear to be enumerated in the presumably vateritic portion and in the zone of transition. These « crystalline » otoliths were rare (0 to 5 % in each experiment), and were discarded. We did not find otoliths wholly crystalline or vateritic.

Analysis of covariance showed that the slopes of the regression lines of fresh weight, standard length and otolith maximum diameter on days elapsed since the beginning of the

experiments (Tables III, IV and V) were significantly different ($P < 0.01$), except those of experiments 1 and 3 ($P > 0.05$). Thus, growth rates of fish and otoliths did not affect the number of major increments. Higher growth rates resulted in wider increments, and not in more increments.

Concerning the regression lines of otolith maximum diameter on fresh weight (Table VI) and on standard length (table VII), analysis of covariance showed that : for the regression of fresh weight the slopes were significantly different ($P < 0.01$), except those of experiments 1 and 3 ($P > 0.05$); for the regressions on standard length the slopes of experiments 5 and 2 differed significantly from those of experiments 1, 3 and 4 ($P < 0.01$), and the slopes were not significantly different within these two groups ($0.01 < P < 0.05$ for comparison between 1 and 4 ; $P > 0.05$ for the other comparisons). Thus, the relationship between otolith size and fish size was affected by the environmental conditions. Similar results were obtained by Marshall & Parker (1982) for *Oncorhynchus nerka*.

Table III. — Characteristics of regression lines of fresh weight (mg) on days elapsed since the beginning of the experiments, and comparison of slopes (Ancova). In this and the following tables : (b) regression coefficients (slopes); (a) y-intercept; (r) correlation coefficients; (n) number of individuals; (F) F ratios; (d.f.) degrees of freedom; (P) probability; F ratios are given only for adjacent experiments.

Experiment	b	a	r	n	F	d.f.	P
3	12.37	-100.44	0.90	39			
1	10.68	- 82.27	0.89	40	1.40	1, 75	> 0.05
4	5.14	- 38.64	0.84	40	28.20	1, 76	< 0.01
2	1.68	- 5.03	0.87	29	27.50	1, 65	< 0.01
5	0.27	4.95	0.54	38	62.79	1, 63	< 0.01

Table IV. — Characteristics of regression lines of standard length (mm) on days elapsed since the beginning of the experiments, and comparison of slopes (Ancova).

Experiment	b	a	r	n	F	d.f.	P
1	0.38	5.17	0.94	40			
3	0.37	5.55	0.93	39	0.20	1, 75	> 0.05
4	0.28	5.01	0.91	40	7.30	1, 75	< 0.01
2	0.14	5.80	0.89	30	27.35	1, 66	< 0.01
5	0.05	5.70	0.69	38	31.45	1, 64	< 0.01

Table V. — Characteristics of regression lines of otolith maximum diameter (μm) on days elapsed since the beginning of the experiments, and comparison of slopes (Ancova).

Experiment	b	a	r	n	F	d.f.	P
3	11.62	114.11	0.98	38			
1	11.40	103.11	0.97	37	0.13	1, 71	> 0.05
4	9.81	94.94	0.97	39	7.13	1, 72	< 0.01
2	7.03	102.92	0.97	30	26.71	1, 65	< 0.01
5	3.26	110.78	0.72	38	30.92	1, 64	< 0.01

Table VI. — Characteristics of regression lines of otolith maximum diameter (μm) on fish fresh weight (mg), and comparison of slopes (Ancova).

Experiment	b	a	r	n	F	d.f.	P
5	8.02	90.81	0.89	38	37.77	1, 63	< 0.01
2	3.49	143.42	0.94	29	30.34	1, 64	< 0.01
4	1.49	193.66	0.91	39	23.61	1, 72	< 0.01
1	0.88	213.93	0.93	37	0.15	1, 71	> 0.05
3	0.85	220.25	0.94	38			

Table VII. — Characteristics of regression lines of otolith maximum diameter (μm) on fish standard length (mm), and comparison of slopes (Ancova).

Experiment	b	a	r	n	F	d.f.	P
5	52.28	-179.42	0.90	38	3.37	1, 64	> 0.05
2	43.02	-130.63	0.96	30	12.58	1, 65	< 0.01
4	31.13	-40.98	0.96	39	1.04	1, 73	> 0.05
3	29.05	-29.05	0.97	38	1.28	1, 71	> 0.05
1	27.05	-17.21	0.97	37			

DISCUSSION

Major increments were formed on a daily basis under all the experimental conditions tested. Photoperiod was not the factor responsible for the periodicity of the increments, that otherwise would not have been deposited daily under constant light (experiments 1, 2 and 3). Temperature was not the determinant factor; since daily increments would only form in experiment 3 (submitted to a circadian rhythm of temperature). Feeding frequency did not determine the periodicity of the increments, since only experiment 2 would induce once increment per day. The number of major increments was not dependent on individual growth rates, which were significantly different. The existence of a circadian endogenous rhythm (biological clock) seems the probable explanation for the deposition of daily microgrowth increments in the lapilli of *T. mariae* juveniles under all the conditions tested, even in the absence of any circadian exogenous stimulus.

Although major increment periodicity was not affected by environmental factors, these had an effect on the characteristics of otolith microstructure, namely on the relative intensities of the dark zones. The increments were more intense in experiments 2 and 4, of intermediate intensity in experiment 5, and less intense in experiments 1 and 3. None of the factors alone seems to explain these observations, since the increments were both intense or faint under constant light, non circadian feeding frequency or non circadian temperature cycle. The hypothesis that the biological clock promotes a circadian cycle of activity or rest, leading to a daily rhythmicity of growth which is reflected in otolith microstructure, could perhaps provide an explanation. It is not established how changes in activity could

affect the material deposited in the otoliths, although Neilson & Geen (1982) suggested that it might be related to activity-induced changes in calcium metabolism. Mugiya *et al.* (1981) showed that there is a daily rhythm in the calcium metabolism of *Carassius auratus*. Pannella (1980) mentioned the existence of a relationship between the intensity of the increments and the nature of the physiological transition between cycles of activity or rest: the increments would be indistinct or faint in species almost constantly active, and well marked in species experiencing rest periods. Our observations of *T. mariae* juveniles revealed that there is an evident effect of each meal on the activity of the individuals, which immediately search for the food particles when they are provided, at least during a light period, even when there are four feedings per day. As the capture of food particles is directed by vision, the meals provided in darkness probably have a different effect on the activity of the juveniles. In experiments 1 (LK, TK, F 1/6) and 3 (LK, TV, F 1/6), the juveniles had a feeding-induced period of activity, or a break in rest, every six hours. The eventual daily period of rest induced by the biological clock would have to be short, being always interrupted by the next meal, and this could be the reason for the faintness of the increments in these experiments. In experiment 2 (LK, TK, F 1/24), the rest period could take more time without interruption (only one meal per every 24 hours) resulting in intense major increments. In experiment 4 (L12:D12, TK, F 1/6) there was one meal per every six hours, but in each day two meals were given during the dark phase, when they probably would not affect the activity or rest of the individuals. The rest period could be relatively long if it occurred during the dark phase, and thus the increments could have intense dark zones. In fact, it was noticed that the juveniles remain inactive close to the bottom when light is turned on after a period of darkness, even during the first seconds of the new light phase. The significantly lower growth rates of the individuals of experiment 4 compared with those of experiments 1 and 3 (Tables III and IV) are an indication that probably the juveniles do not eat during the dark phase. Concerning experiment 5 (L12:D12, TK, F 1/48), the shortage of food could be responsible for the narrowness of the increments (growth rates were very low in this experiment), and so for their relative faintness.

Neilson & Geen (1982) concluded that feeding frequency interacts with the endogenous rhythm responsible for increment production in *Oncorhynchus tshawytscha*, which can result in deviations from the one increment/24 h relationship. The indirect influence of feeding periodicity on the intensity of the increments can perhaps come to the point of inhibiting the action of the endogenous rhythm in *T. mariae*, for example under higher feeding frequencies. Some authors have concluded that a 24 hours light and dark cycle is needed to promote the action of the circadian endogenous rhythm (Taubert & Coble, 1977; Tanaka *et al.*, 1981). It is possible that this also happens in *T. mariae*, as the embryos and larvae were reared under a L12:D12 photoperiod, which could have entrained the endogenous rhythm. Our preliminary observations of the lapilli of some larvae kept under constant darkness suggest that no increments are formed under these conditions, or that they are less numerous than the number of days. This would agree with Radtke & Dean

(1982) results in *Fundulus heteroclitus* and would show an influence of light on the entrainment of the biological clock.

Although we did not make counts of minor increments total numbers, we estimated their number per major increment in the different experiments. In experiments 1 and 3 major increments were often divided into four minor ones, suggesting a direct relationship between the number of meals and the number of subdaily increments. In experiment 4, although there were four meals per day, two or three minor increments per major increment were formed, which could be explained by the fact that two of the daily meals were given during the dark phase. In experiment 2, minor increments, when present, were also two or three per major increment, an explanation for their formation being less clear. A relationship between this number and the unknown number of short temperature cycles determined by the thermostats on a non daily basis might be possible; this would agree with Brothers (1981) conclusions. In experiment 5, subdaily increments were almost completely inexistent, probably due to the narrowness of the major increments. There seems to be a relationship between feeding periodicity and the number of subdaily increments, as has also been suggested by Campana (1983). This relationship could be indirect, if we assume that the growth rate of the individuals, which is influenced by feeding, is responsible for the number of subdaily increments. Another feature of otolith microstructure which is influenced by feeding periodicity is the intensity of the dark zones, as shown by the subtle bidaily pattern observed in otoliths of juveniles fed once every 48 h (experiments 5 and 2-A). There is evidence that the endolymph composition is directly influenced by feeding and that the formation of a zone containing more protein coincides with an increase in the protein contents of the endolymph (Love, 1980). Thus, this can be the reason why the presence or absence of food in each day can directly influence the intensity of the dark zone deposited. Campana (1983) showed that the influence of feeding on subdaily increments number and on intensity patterns is probably dependent on a high metabolic rate, which is probably the case of *T. mariae*.

Under the rearing conditions described, *T. mariae* larvae started depositing increments in their lapilli on day 2 after hatching, and major increments were formed on a recognizable daily basis since swimming-up. It seems that the circadian endogenous rhythm starts functioning only some days after hatching, when the larvae start swimming, which might be related to a change in their activity/rest patterns. The end of endogenous feeding seems the probable reason for the more regular daily increments after yolk-sac absorption.

Our results have pointed out the existence of a circadian endogenous rhythm controlling the formation of daily increments in the lapilli of *T. mariae* juveniles, perhaps by determining a daily cycle of activity or rest. Feeding frequency and photoperiod influenced the characteristics of otolith microstructure, probably by interfering with the action of the endogenous rhythm. Our conclusions are not necessarily in contradiction with those of several other authors (Taubert & Coble, 1977; Tanaka *et al.*, 1981; Neilson & Geen, 1982; Radkte & Dean, 1982; Marshall & Parker, 1982; Campana, 1983). Also, they would explain the formation of daily incre-

ments in wild fish under all environmental conditions including in deep-living species (Brothers *et al.*, 1976) and in fish from above the Arctic Circle (Townsend & Shaw, 1982). The formation of daily increments seems to be a widespread characteristic of otolith growth, and otolith composition is remarkably uniform independently of the species and of the habitat (Degens *et al.*, 1969); due to these reasons, the existence of very different explanations for the deposition of daily increments in fish otoliths is not probable. Some of the divergent results may have an origin on the use of different ontogenic stages and ages, which can be susceptible in different ways to the action of exogenous factors. Contradictory results can perhaps be due in some cases to difficulties in the distinction of daily and subdaily increments. The existence of a biological clock, more or less affected by environmental factors, seems to be the conclusion reached most often. More investigation with different species, ontogenic stages and environmental conditions will be needed before we understand better the physiology of otolith growth.

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